Letter

D-alanylation in the assembly of ansatrienin side chain is catalyzed by a modular NRPS

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D-alanylation in the assembly of ansatrienin side chain is catalyzed by a modular NRPS

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Abstract

Ansatrienins are a group of ansamycins with *N*-cyclohexanoyl D-alanyl side chain. Though ansatrienins have been identified for decades, the mechanism for the addition of this unique side chain were not established. Here, we report the biochemical characterization of a tridomain nonribosomal peptide synthetase (NRPS), AstC, and an *N*-acyltransferase, AstF1, encoded in the biosynthetic pathway of ansatrienins. We demonstrate that AstC can efficiently catalyze the transfer of D-alanine to the C-11 hydroxyl group of ansatrienins, and AstF1 is able to attach the cyclohexanoyl group to the amino group of D-alanine. Remarkably, AstC presents the first example that a modular NRPS can catalyze intermolecular D-alanylation of hydroxyl group to form an ester bond, though alanyl natural products have been known for decades. In addition, both of AstC and AstF1 have broad substrate specificity towards acyl donors, which can be utilized to create novel ansatrienins.

D-alanylation is of broad biological significance as exemplified by that of teichoic acids (TAs), major components of the low-G+C Gram-positive cell wall. D-alanylation of TAs is involved in diverse physiological process, including antibiotic resistance, biofilm formation, virulence, and acid tolerance.[1] The modification of TAs with D-alanine involves enzymes encoded by the *dltABCD* operon, and the whole pathway displays remarkable parallelism to the division of labor employed by nonribosomal peptide synthetases (NRPSs).[2] Similar pathways for the glycylation of lipopolysaccharide in Gram-negative bacteria had also been identified.[3] However, although D-alanylated small-molecule natural products have been identified for decades, the biosynthetic mechanism of this modification has not been established.

Ansatrienins belong to a unique group of ansamycin antibiotics produced by *Streptomyces*.[4] These small molecules contain a 21-membered macrocyclic lactam ring and a cyclohexanoyl-alanyl side chain attached to the C-11 hydroxyl group of *ansa* ring. The biosynthesis of ansatrienins is presumed to follow the same manner as suggested for other ansamycins, which are produced by multimodular type I polyketide synthases (PKSs) using 3-amino-5-hydroxybenzoic acid (AHBA) as the starter unit, and released by amide synthases followed by post-PKS modifications.[5] Although the biosynthetic gene clusters for ansatrienins have been reported,[6, 7] the attachment of the cyclohexanoyl-alanyl side chain has not been revealed.

Previously, feeding experiments with *Streptomyces collinus* Tü 1892 have demonstrated that the D-alanine moiety of ansatrienins is derived directly from free D-alanine rather than L-alanine, and cyclohexanoyl-alanyl side chain is not incorporated as an intact moiety but added one component at a time.[8] On the basis of bioinformatic analysis of the *myc* gene cluster in *Streptomyces flaveolus* DSM 9954, Qu *et al*. proposed that the NRPS MycC is involved in adenylating alanine residue, which is then transferred from MycC to the C-11 hydroxyl group of *ansa* ring by the esterase MycF4, followed by *N*-cyclohexyl formylation catalyzed by the *N*-acetyltransferase MycF1.[7] Recently, as part of

our efforts to exploit novel ansamycins from AHBA synthase gene positive strains,[9-11] an ansatrienin gene cluster (*ast*, GenBank accession number: KF813023.1 and KP284551) was activated by constitutive expression of the pathway-specific positive regulator gene *astG1* in *Streptomyces* sp. XZQH13, resulting in the isolation of two known ansatrienins, hydroxymycotrienin A (**1**) and thiazinotrienomycin G (**2**) (Figure 1a).[12] However, sequence analysis revealed that the homolog of *mycF4* gene was absent in the *ast* gene cluster, suggesting that MycF4 or its homologs may be not required for the transfer of the alanine residue. Accordingly, an isolate tridomain (adenylation-thiolation-thioesterase, A-T-TE) NRPS, namely AstC, a homolog of MycC, was speculated to be responsible for the attachment of D-alanyl group to the *ansa* ring. This type of A-T-TE-tridomain NRPS-mediated intermolecular amino acid esterification is unprecedented among known esterases or NRPSs. Here we demonstrate that *astC* and *astF1* are essential genes for the attachment of the cyclohexanoyl-alanyl side chain onto ansatrienin *ansa* ring, AstC can catalyze the attachment of D-alanine into the biosynthetic intermediate (**3**) (Figure 1a), and AstF1 is responsible for the attachment of the cyclohexanoyl moiety.

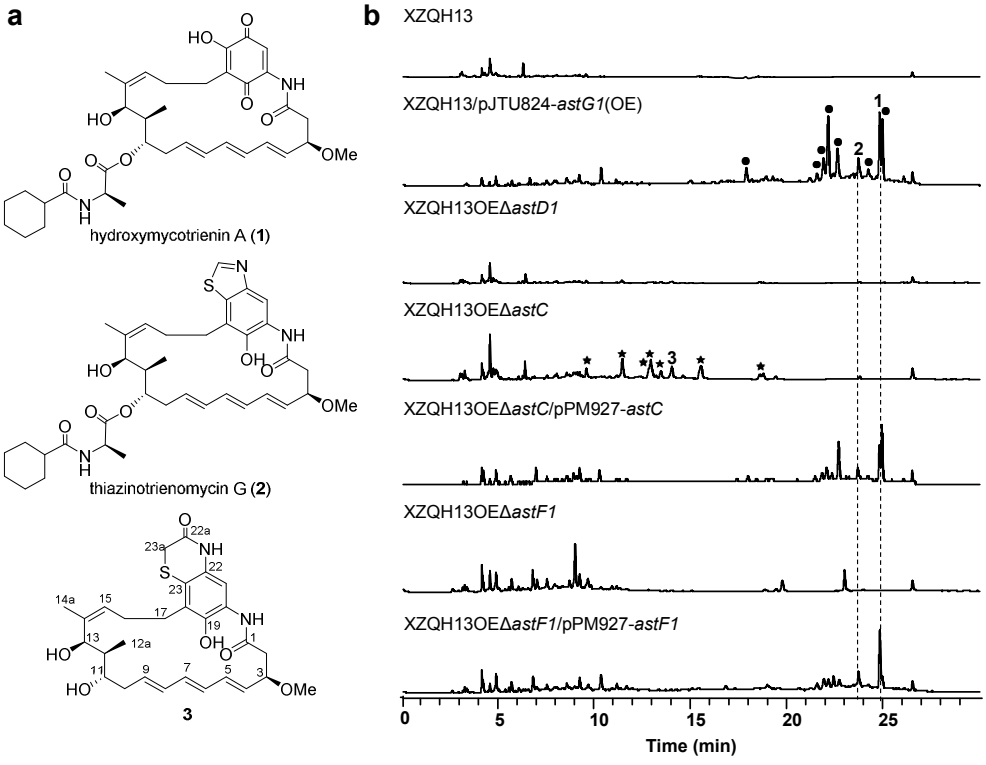

Figure 1. The *astC* and *astF1* genes are required for the attachment of the cyclohexanoyl-alanyl side chain onto ansatrienin *ansa* ring in *Streptomyces* sp. XZQH13/pJTU824-*astG1*(OE). a) Structures of the representative ansatrienins isolated from the strain XZQH13. b) HPLC analysis (275 nm) of metabolites produced by the strain XZQH13 and the corresponding mutant strains. Uncharacterized ansatrienin analogs were identified by diode array UV comparison with that compounds **1**–**3** in the strains XZQH13/pJTU824-*astG1*(OE) (●) and XZQH13OEΔ*astC* (★), respectively. It is worth noting that the intermediates accumulated by XZQH13OEΔ*astF1* are refractory to isolations thus that we have not obtained enough amount for full structure elucidation.

The production of ansatrienins is low in the strain XZQH13 but increased tremendously by overexpressing the regulator gene *astG1* thus that many uncharacterized extra peaks were presented in the mutant XZQH13/pJTU824-*astG1*(OE) (Figure 1b).[12] All these extra peaks were identified to be ansatrienins by HPLC comparison of the fermentation products of the PKS gene (*astD1*)-disrupted mutant XZQH13OEΔ*astD1*, and XZQH13/pJTU824-*astG1*(OE) with that of ansatrienins (Figure 1b , Supplementary Figure S4), supporting that the *ast* gene cluster is responsible for the biosynthesis of ansatrienins. To confirm the roles of *astC* and *astF1* in the incorporation of the cyclohexanoyl-alanyl side chain, the individual gene-disrupted mutants were generated and complemented *in trans* of a full-length copy of the disrupted gene under the control of the promoter of the erythromycin resistance gene (*ermE**) (Supplementary Figure S5). Both of the resultant mutants XZQH13OEΔ*astC* and XZQH13OEΔ*astF1* abolished the production of compounds **1** and **2** along with the uncharacterized analogs, and recovered the production of ansatrienins after complementing the *astC* and *astF1* genes (Figure 1b), respectively, indicating that both genes are required for the attachment of the cyclohexanoyl-alanyl side chain onto ansatrienin *ansa* ring.

To investigate the function of AstC, we identified the metabolic intermediates accumulated in XZQH13OEΔ*astC*. Compound **3** was obtained

from the fermentation products of XZQH13OEΔ*astC* (Figure 1a). Its high-resolution mass spectrometry (HRMS) showed a *quasi* molecular ion at *m/z* 527.2152 for **3** [M($C_{28}H_{35}N_2O_6S$) – H]$^-$ (Supplementary Figure S11). Its structure was established by the NMR comparison with that of thiazinotrienomycin E (Supplementary Table S3, Supplementary Figures S7 – 10).[13] The NMR spectroscopic data of both compounds were similar, except for the absence of the cyclohexanoyl-alanyl side chain at C-11 in **3**, which indicated that the *astC* gene may be involved in the incorporation of the D-alanine residue in ansatrienins.

The *astC* gene encodes a single-module A-T-TE-tridomain NRPS-like protein. The closest homologs in NCBI are AnsC and MycC (protein accession numbers AHW80292.1 and AFG19416.1) of *S. seoulensis* and *S. flaveolus* DSM 9954, respectively, both shared 88% identity of amino acid sequences with AstC. However, none of these two proteins have been functionally characterized. To obtain direct evidence for the role of AstC, we amplified the 2541-bp fragment of *astC* gene using PCR and cloned it into pET28a(+) for expression in *E. coli* BL21(DE3). To produce the *holo*-enzyme, heterologously expressed AstC was treated *in vitro* with the phosphopantetheinyl transferase Sfp. The activity of *holo*-AstC was assayed using compound **3** as acyl acceptor and D/L-alanine as acyl donors. LC-MS analysis showed the presence of an extra peak (**4**) with a *quasi* molecular ion at *m/z* 598.2515 [M – H]$^-$ (Figure 2a, Supplementary Figure S12), in accordance with the attachment of an alanyl residue to **3**, in the reaction with D-alanine. In contrast, reactions either with boiled AstC or L-alanine did not afford any evident products (Figure 2a). These results show that AstC catalyzes the alanylation of compound **3** with D-alanine but not L-alanine as the acyl donor to produce compound **4** (Figure 2c), which is consistent with the selective use of D-alanine in feeding experiments.[8]

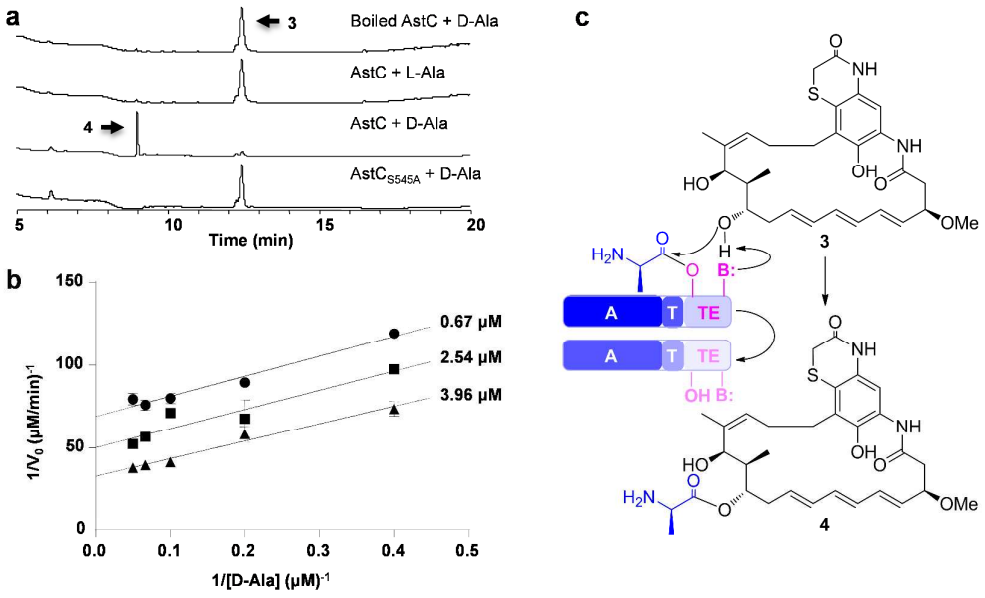

Figure 2. Reconstitution of AstC activity in vitro. a) HPLC traces (260 nm) the incubations of L-/D-alanine and **3** with *holo*-AstC and *holo*-AstC$_{S545A}$. b) AstC follows the "ping-pong" mechanism. c) AstC was proposed to incorporate the D-alanine residue at C-11 of **3**.

To characterize the enzymatic properties, the *holo*-AstC-catalyzed reaction was quantified by HPLC. The purified *holo*-AstC showed optimal activity at pH 8.0, and exhibited more than 75% of its maximum activity between pH 7.0 and 10.0 (Supplementary Figure S3a). The optimal reaction temperature was 30 °C at pH 8.0, with > 75% activity between 25 and 35 °C (Supplementary Figure S3b). The kinetic parameters of *holo*-AstC were measured under the optimal conditions, showing the conversion with an apparent K_M of 40.21 ± 20.60 μM and k_{cat} of 0.70 ± 0.20 min^{-1} for compound **3**, and an apparent K_M of 5.57 ± 1.56 μM and k_{cat} of 0.27 ± 0.03 s^{-1} for D-alanine (Supplementary Figures S3d, e). To determine the mechanism of reaction catalyzed by AstC, the concentrations of D-alanine were varied with different constant concentrations of compound **3**. When plotting the reciprocal of V_0 *versus* the reciprocal of the D-alanine concentrations, parallel lines were obtained (Figure 2b), showing that AstC transfers the D-alanyl to the C-11 hydroxyl group of **3** via the "ping-pong" mechanism.

To address the substrate specificity of AstC, sodium propionate, *β*-alanine, glycine and 12 D-amino acids were selected as acyl donors. The strongest conversion was detected with D-alanine, followed by D-serine, *β*-alanine, glycine, D-threonine, and D-valine (**4**–**9**; Supplementary Figure S6). The other tested D-amino acids were not accepted at the detected levels. This relaxed substrate specificity toward acyl donors suggests a rather non-specific activation of amino acids by the A-domain of AstC, which will allow for chemoenzymatic synthesis of new ansatrienin analogs. We also assayed the tolerance of AstC toward different acyl acceptors by performing the acylation assay with various ansamycins, including *N*-desmethyl-4,5-desepoxymaytansinol,[14] geldanamycin and hygrocins.[15] However, no positive results were obtained (data not shown), suggesting a strict substrate specificity of AstC for the acyl acceptors.

The notable feature of the AstC is the presence of only three individual domains (A-T-TE) responsible for adenylation, thiolation and thioester cleavage, and the absence of the traditional condensation (C) domain. Previously, only a few such A-T-TE-tridomain, one module NRPS proteins have been characterized, all are involved in the homodimerizations of aromatic *α*-keto acids by catalyzing carbon-carbon bond formation.[16-18] AstC represents a new member of A-T-TE-tridomain NRPS enzymes but with quite different function.[19, 20] The D-alanylation catalyzed by AstC presents a unique acylation strategy, which differs markedly from any other acyltransferases.[21, 22] In the latter biosynthetic manifolds, the enzyme-mediated acylations usually require coenzyme A (CoA) thioesters or *N*-acetylcysteamine (NAC) thioesters as substrates to produce the acylated products.[23] By contrast, AstC utilizes free amino acids as acyl donors that are activated as aminoacyl adenylates by the A-domain, and then transferred to the T-domain, transiently forming aminoacyl thioesters. Indeed, when the active site Ser545 within the conserved GGXSXLA motif of the T-domain was mutated to alanine, the mutant AstC was no longer capable of generating **4** from **3** (Figure 2a). Transfer of the activated acyl moiety from the T-domain to the TE-domain would form the acyl-*O*-TE enzyme intermediate (Figure 2c). At this point, we proposed that the acyl carbonyl carbon of this intermediate was

nucleophilically attacked by the C-11 hydroxyl oxygen of **3** to complete the acylation (Figure 2c). However, it is not yet clear how AstC recognizes and interacts with the acyl acceptor, **3**. Moreover, so far, only a PCP-C-didomain (Fum14) and a freestanding C-domain (SgcC5) NRPS-like proteins have been biochemically characterized to use free alcohols as the acyl acceptors to couple with acyl carrier protein-tethered acyl donors for ester bond formations.[24-26] To our knowledge, AstC is the first example of an A-T-TE-tridomain NRPS protein that is capable of catalyzing intermolecular ester bond formation, particularly, using free amino acids as acyl donors.

AstF1 identified as an acyltransferase was suggested to catalyze the transfer of cyclohexanoyl moiety to the alanyl nitrogen at C-11 of ansatrienins.[7] To establish the function of AstF1, the *astF1* gene (927 bp) was amplified from fosmid 17-10F by PCR, and inserted into pET22b for expression in *E. coli* BL21(DE3). Due to lack of the intermediates accumulated by the *astF1*-deleted mutant (XZQH13OEΔ*astF1*), we directly examined whether compound **4** can be used as the acyl acceptor in the transacyl reaction. Incubation of the recombinant AstF1 protein with the reaction mixture of AstC assay containing compound **4** and the chemically synthesized cyclohexanoyl-SNAC (**I**) (Figure 3a) led to the formation of thiazinotrienomycin E (**10**) accompanied by the decrease of **4** (Figure 3b). To confirm this result, an identical incubation without AstF1 was conducted; no conversion of **4** was detected. These results clearly demonstrated the predicted acyltransferase activity of AstF1 (Figure 3a).

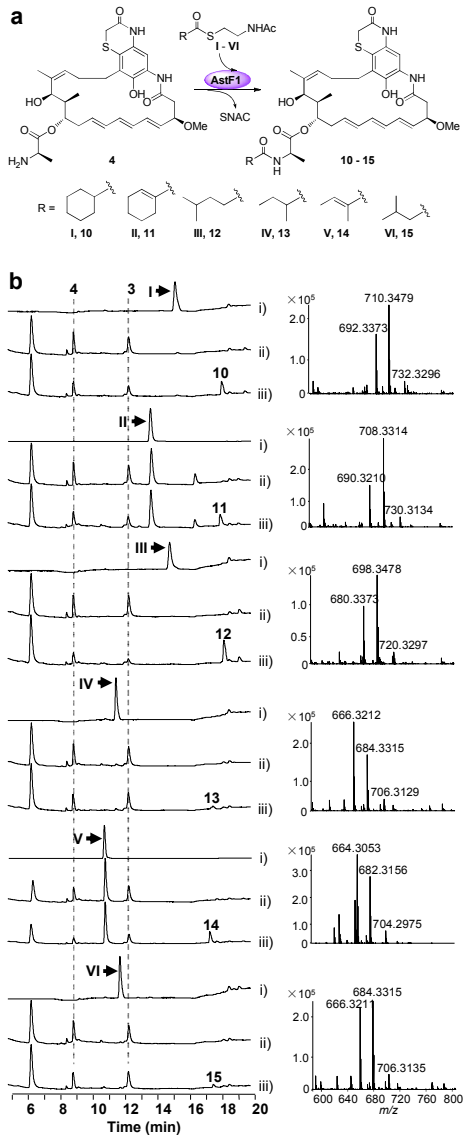

Figure 3. Reconstitution of AstF1 activity in vitro. a) The reactions catalyzed by AstF1. b) LC-MS analysis of the reaction products **10**–**15** of AstF1 incubated with *N*-acetylcysteamine (SNAC) thioesters **I**–**VI** and **4** (produced by the incubation of **3** and D-alanine with *holo*-AstC), respectively. i), SNAC thioesters; ii), SNAC thioesters and **4** without AstF1; iii), SNAC thioesters and **4** with AstF1.

Broad substrate specificity with regard to the acyl donors of acyltransferases has been commonly observed and utilized to generate analogs of natural products with varied acyl substituents.[27] We examined whether cyclohex-1-enecarbonyl-SNAC (**II**), 4-methylpentanoyl-SNAC (**III**), 2-

methylbutanoyl-SNAC (**IV**), 2-methylbut-2-enoyl-SNAC (**V**), and 3-methylbutanoyl-SNAC (**VI**) could replace **I** as the acyl donors in the acylations of **4**. As expected, all these SNAC thioesters can serve as substrates for AstF1, but with different turnover rates, to produce **11** – **15** (Figure 3b). The demonstrated ability of the recombinant AstF1 to use various acyl-SNAC substrates is consistent with the substitution profile of identified natural ansatrienins. The mutasynthesis study by Song *et al*. has also shown that a diversity of small molecule organic acids can be attached to the D-alanyl nitrogen of ansatrienins, which is supposed to be catalyzed by a homolog of AstF1 in *S. seoulensis* IFB-A01.[28] Therefore, the remarkable substrate tolerance of AstF1 to acyl donors provides an efficient chemoenzymatic route to new ansatrienin analogs.

In conclusion, we have confirmed that the A-T-TE-tridomain NRPS protein AstC catalyzes the attachment of D-alanyl to the C-11 hydroxyl group of ansatrienin backbone, which represents an unprecedented acylation mechanism, and AstF1 catalyzes the sequential attachment of the cyclohexanoyl group to the D-alanyl nitrogen during ansatrienins biosynthesis. Moreover, kinetic studies of the D-alanyl transfer reaction catalyzed by AstC demonstrated that the reaction occurred by the “ping-pong” mechanism. In addition, both of AstC and AstF1 display broad substrate specificity toward the acyl donors. The ability to decorate the biosynthetic intermediates of ansatrienins with combinations of various amino acids and small organic acids via the actions of AstC and AstF1 should provide routes to new variants in the ansamycin class.

Methods

Inactivation of *astD1*, *astF1* and *astC* in XZQH13/pJTU824-*astG1*

The genomic library of *Streptomyces* sp. XZQH13 was constructed and screened by PCR using AHBA synthase-specific degenerate primers to identify fosmid 17-10F. The target genes in fosmid 17-10F were replaced by the apramycin resistance cassettes (*acc(3)IV(Apr^R)* + *oriT*) by λ-RED

mediated PCR-targeting method. The mutant fosmids were introduced into *E. coli* ET12567/pUZ8002 for conjugation with XZQH13/pJTU824-*astG1*(OE). Apramycin resistant exconjugants were selected and verified by PCR to generate XZQH13OEΔ*astD1*, XZQH13OEΔ*astC* and XZQH13OEΔ*astF1*, respectively (Supplementary Figure S4).

In vitro assays for AstC and $AstC_{S545A}$

The *holo*-AstC and *holo*-$AstC_{S545A}$ were obtained by incubating *apo*-AstC or *apo*-$AstC_{S545A}$ (2.5 μM) and CoA (100 μM) with Sfp (1 μM) in a 50 μL reaction containing Tris-HCl (50 mM, pH 8.0), $MgCl_2$ (10 mM), and DTT (1 mM). After 1 h incubation at 30 °C to prime the T-domain, the reaction was initiated by the addition of ATP (2 mM), amino acids (1 mM), and compound **3** (26 μM). After incubation for 2 h at 30 °C, a 100 μL reaction was quenched by the addition of 100 μL methanol and centrifuged for 10 min at 13,000 rpm. The supernatant was analyzed by HPLC (Supporting Information). The details of characterization of AstC see Supporting Information.

In vitro assay for AstF1

Six acyl-SNACs were synthesized as the substrates for AstF1 (Supporting Information). The reaction mixture of formation of compound **4** contained Tris-HCl (50 mM, pH 8.0), $MgCl_2$ (10 mM), *holo*-AstC (2.5 μM), compound **3** (26 μM), D-alanine (1 mM), ATP (2 mM), and DTT (1 mM). After incubation for 2 h at 30 °C to synthesize **4**, the reaction was initiated by the addition of acyl-SNAC (2 mM), glycerol (5%), AstF1 (5 μM) and DTT (1 mM). Reactions without AstF1 were served as the control. After incubation for 5 h at 30 °C, a 75 μL reaction was quenched by the addition of 75μL methanol and centrifuged for 10 min at 13,000 rpm. The supernatant was analyzed by LC-MS similar as that for AstC, but in positive mode (Supporting Information).

Associated Content

Supporting Information

The isolation of compound **3**, the synthesis of acyl-SNACs, Tables S1–3, and Figures S1–30 are available free of charge via the Internet from http://pubs.acs.org.

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Notes

The authors declare no competing financial interests.

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References

(1) Santa Maria, J. P., Jr., Sadaka, A., Moussa, S. H., Brown, S., Zhang, Y. J., Rubin, E. J., Gilmore, M. S., and Walker, S. (2014) Compound-gene interaction mapping reveals distinct roles for *Staphylococcus aureus* teichoic acids, *Proc. Natl. Acad. Sci. U S A 111*, 12510–12515.

(2) Roy, H. (2009) Tuning the properties of the bacterial membrane with aminoacylated phosphatidylglycerol, *IUBMB Life 61*, 940–953.

(3) Henderson, J. C., Fage, C. D., Cannon, J. R., Brodbelt, J. S., Keatinge-Clay, A. T., and Trent, M. S. (2014) Antimicrobial peptide resistance of *Vibrio cholerae* results from an LPS modification pathway related to nonribosomal peptide synthetases, *ACS Chem. Biol. 9*, 2382–2392.

(4) Sugita, M., Sasaki, T., Furihata, K., Seto, H., and Otake, N. (1982) Studies on mycotrienin antibiotics, a novel class of ansamycins. II. structure elucidation and biosynthesis of mycotrienins I and II, *J. Antibiot. 35*, 1467–1473.

(5) Kang, Q., Shen, Y., and Bai, L. (2012) Biosynthesis of 3,5-AHBA-derived natural products, *Nat. Prod. Rep. 29*, 243–263.

(6) Chen, S., von Bamberg, D., Hale, V., Breuer, M., Hardt, B., Muller, R., Floss, H. G., Reynolds, K. A., and Leistner, E. (1999) Biosynthesis of ansatrienin (mycotrienin) and naphthomycin. Identification and analysis of two separate

biosynthetic gene clusters in *Streptomyces collinus* Tu 1892, *Eur. J. Biochem. 261*, 98–107.

(7) Qu, X., Lei, C., and Liu, W. (2011) Transcriptome mining of active biosynthetic pathways and their associated products in *Streptomyces flaveolus*, *Angew. Chem. Int. Ed. 50*, 9651–9654.

(8) Wu, T. S., Duncan, J., Tsao, S. W., Chang, C. J., Keller, P. J., and Floss, H. G. (1987) Biosynthesis of the ansamycin antibiotic ansatrienin (mycotrienin) by *Streptomyces collinus*, *J. Nat. Prod. 50*, 108–118.

(9) Wang, H., Chen, Y., Ge, L., Fang, T., Meng, J., Liu, Z., Fang, X., Ni, S., Lin, C., Wu, Y., Wang, M., Shi, N., He, H., Hong, K., and Shen, Y. (2013) PCR screening reveals considerable unexploited biosynthetic potential of ansamycins and a mysterious family of AHBA-containing natural products in actinomycetes, *J. Appl. Microbiol. 115*, 77–85.

(10) Li, S., Li, Y., Lu, C., Zhang, J., Zhu, J., Wang, H., and Shen, Y. (2015) Activating a cryptic ansamycin biosynthetic gene cluster to produce three new naphthalenic octaketide ansamycins with *n*-pentyl and *n*-butyl side chains, *Org. Lett. 17*, 3706–3709.

(11) Zhang, J., Qian, Z., Wu, X., Ding, Y., Li, J., Lu, C., and Shen, Y. (2014) Juanlimycins A and B, ansamycin macrodilactams from *Streptomyces* sp., *Org. Lett. 16*, 2752–2755.

(12) Xie, C., Deng, J., and Wang, H. (2015) Identification of AstG1, a LAL family regulator that positively controls ansatrienins production in *Streptomyces* sp. XZQH13, *Curr. Microbiol. 70*, 859–864.

(13) Smith, A. B., Barbosa, J., Hosokawa, N., Naganawa, H., and Takeuchi, T. (1998) (+)-Thiazinotrienomycin E: relative and absolute stereochemistry, *Tetrahedron Lett. 39*, 2891–2894.

(14) Li, S., Lu, C., Chang, X., and Shen, Y. (2015) Constitutive overexpression of *asm18* increases the production and diversity of maytansinoids in *Actinosynnema pretiosum*, *Appl. Microbiol. Biotechnol.*, Epub ahead of print (DOI: 10.1007/s00253-00015-07127-00257).

(15) Lu, C., Li, Y., Deng, J., Li, S., Shen, Y., Wang, H., and Shen, Y. (2013) Hygrocins C-G, cytotoxic naphthoquinone ansamycins from *gdmAI*-disrupted *Streptomyces* sp. LZ35, *J. Nat. Prod. 76*, 2175–2179.

(16) Wackler, B., Schneider, P., Jacobs, J. M., Pauly, J., Allen, C., Nett, M., and Hoffmeister, D. (2011) Ralfuranone biosynthesis in *Ralstonia solanacearum* suggests functional divergence in the quinone synthetase family of enzymes, *Chem. Biol. 18*, 354–360.

(17) Balibar, C. J., Howard-Jones, A. R., and Walsh, C. T. (2007) Terrequinone A biosynthesis through L-tryptophan oxidation, dimerization and bisprenylation, *Nat. Chem. Biol. 3*, 584–592.

(18) Zhu, J., Chen, W., Li, Y. Y., Deng, J. J., Zhu, D. Y., Duan, J., Liu, Y., Shi, G. Y., Xie, C., Wang, H. X., and Shen, Y. M. (2014) Identification and catalytic characterization of a nonribosomal peptide synthetase-like (NRPS-like) enzyme involved in the biosynthesis of echosides from *Streptomyces* sp. LZ35, *Gene 546*, 352–358.

(19) Ishikawa, F., Konno, S., Suzuki, T., Dohmae, N., and Kakeya, H. (2015) Profiling nonribosomal peptide synthetase activities using chemical proteomic probes for adenylation domains, *ACS Chem. Biol. 10*, 1989–1997.

(20) Ishikawa, F., Miyamoto, K., Konno, S., Kasai, S., and Kakeya, H. (2015) Accurate detection of adenylation domain functions in nonribosomal peptide synthetases by an enzyme-linked immunosorbent assay system using active site-directed probes for adenylation domains, *ACS Chem. Biol. 10*, 2816–2826.

(21) Wang, F., Wang, Y., Ji, J., Zhou, Z., Yu, J., Zhu, H., Su, Z., Zhang, L., and Zheng, J. (2015) Structural and functional analysis of the loading acyltransferase from avermectin modular polyketide synthase, *ACS Chem. Biol. 10*, 1017–1025.

(22) Gay, D. C., Spear, P. J., and Keatinge-Clay, A. T. (2014) A double-hotdog with a new trick: structure and mechanism of the trans-acyltransferase polyketide synthase enoyl-isomerase, *ACS Chem. Biol. 9*, 2374–2381.

(23) Koryakina, I., McArthur, J., Randall, S., Draelos, M. M., Musiol, E. M., Muddiman, D. C., Weber, T., and Williams, G. J. (2013) Poly specific trans-acyltransferase machinery revealed via engineered acyl-CoA synthetases, *ACS Chem. Biol. 8*, 200–208.

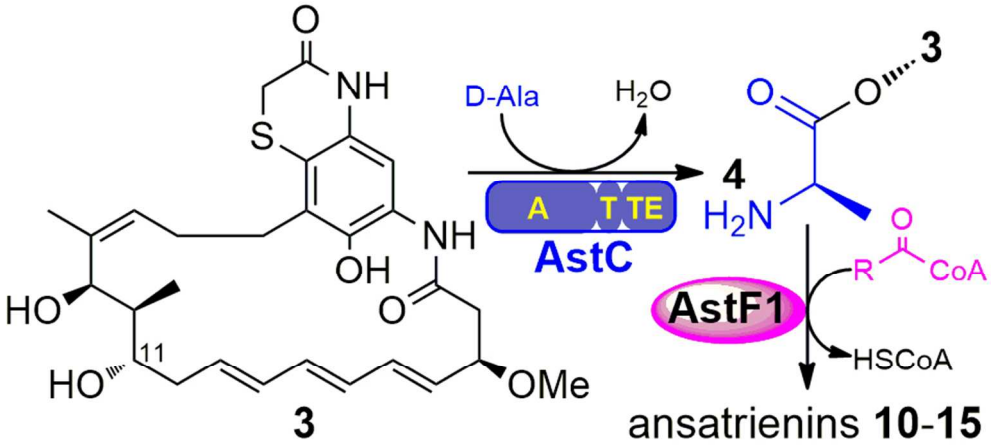
(24) Zaleta-Rivera, K., Xu, C., Yu, F., Butchko, R. A., Proctor, R. H., Hidalgo-Lara, M. E., Raza, A., Dussault, P. H., and Du, L. (2006) A bidomain nonribosomal peptide synthetase encoded by *FUM14* catalyzes the formation of tricarballylic esters in the biosynthesis of fumonisins, *Biochemistry 45*, 2561–2569.

(25) Lin, S., Van Lanen, S. G., and Shen, B. (2009) A free-standing condensation enzyme catalyzing ester bond formation in C-1027 biosynthesis, *Proc. Natl. Acad. Sci. U S A 106*, 4183–4188.

(26) Lin, S., Huang, T., Horsman, G. P., Huang, S.-X., Guo, X., and Shen, B. (2012) Specificity of the ester bond forming condensation enzyme SgcC5 in C-1027 biosynthesis, *Org. Lett. 14*, 2300–2303.

(27) Moss, S. J., Bai, L., Toelzer, S., Carroll, B. J., Mahmud, T., Yu, T. W., and Floss, H. G. (2002) Identification of Asm19 as an acyltransferase attaching the biologically essential ester side chain of ansamitocins using *N*-desmethyl-4,5-desepoxymaytansinol, not maytansinol, as its substrate, *J. Am. Chem. Soc. 124*, 6544–6545.

(28) Song, Y. N., Jiao, R. H., Zhang, W. J., Zhao, G. Y., Dou, H., Jiang, R., Zhang, A. H., Hou, Y. Y., Bi, S. F., Ge, H. M., and Tan, R. X. (2015) New ansamycin derivatives generated by simultaneous mutasynthesis, *Org. Lett. 17*, 556–559.



The A-T-TE-tridomain NRPS protein AstC catalyzes the D-alanylation of C-11 hydroxyl group, and the acyltransferase AstF1 catalyzes the sequential attachment of acyl group to the D-alanyl nitrogen during the biosynthesis of ansatrienins.
81x37mm (300 x 300 DPI)